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and Its Prognostic/Predictive Value

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14. ABSTRACT There is rising biologic concern in breast cancer that the current standard purely measures nuclear ER expression, despite the fact that it is now widely accepted amongst scientists that ER signals non-genomically. The ultimate goal of this research is to develop one (or a variety of) assay(s) that can measure this non-genomic signaling and provide superior value in predicting response to Tamoxifen. My results to date have allowed me to develop a quantified and reproducible assay to measure nuclear ER, and use this assay to assess the rate and significance of misclassification in breast cancer patients today. We have also been able to detect non-nuclear ER in clinical samples, confirming that it is bona-fide ER, and that it appears to correlate with worse patient prognosis. We are also developing an assay to multiplex Src with full-length ER and determine whether this can provide additional prognostic or predictive value.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	9
Reportable Outcomes.....	9
Conclusion.....	10
References.....	10
Appendices.....	12

INTRODUCTION:

The ultimate goal of this research is to develop one (or a variety of) assay(s) that can provide superior value in predicting response to Tamoxifen. This goal was motivated by the main biologic concern that the current standard purely measures nuclear ER expression, despite the fact that it is now widely accepted amongst scientists that ER signals non-genomically. This non-genomic signaling underlies Tamoxifen resistance in many preclinical models (1-5), and can involve full-length receptor or shorter isoforms (6-9), as well as cross-talk with other GFRs (10-12) and cytoplasmic kinase pathways (13-14). Therefore, the focus of this research was on developing assays which can measure different aspects of this non-genomic signaling. I chose to focus my proposal on non-nuclear ER and its cross-talk with Src, since this is a major component of non-genomic signaling (1, 12, 15-18).

BODY:

The original statement of work was the following:

Build an assay to quantitatively assess the activity of non-genomic ER α signaling in breast cancer

Task 1 Construct cell line models for genomic and non-genomic pathways

Methods: Culture MCF7 (genomic model), MCF-7/HER2-18 cells (non-genomic model); cell stimulation (E2, EGF, IGF-1, tamoxifen, EDC) and IB for ER α (in non-nuclear fraction of lysate), pER α , pHER2 (all within minutes); and ERE-gene reporter assay (within hours)

Timeline: Months 1-7

Outcomes/Deliverables: A cell line model displaying high levels of non-genomic ER α signaling (MCF7/HER2-18), as well as one displaying low levels (but high genomic signaling) as a negative control.

Task 2 Validate and develop antibodies to best distinguish activity of the non-genomic pathway

Methods: Selected antibodies, both cell lines: Subcellular fractionation and subsequent IB for ER α ; Immunofluorescence (IF) on coverslips (for ER α , Src, active Src); image capture with DeltaVision microscope; co-IP (ER α /Src); IB for pMAPK; reporter assays (ERE, ERK genes); siRNA to ER α , Src; cell line array construction; IF on cell line array (for ER α , Src, active Src); Image capture with PM-200 fluorescence microscope, AQUA analysis

Timeline: Months 4-20

Outcomes/Deliverables: A quantitative non-genomic ER α pathway assay: validated antibodies and IF readouts of non-genomic signaling measured with AQUA

Determine the prognostic and predictive value of the non-genomic ER α pathway assay in breast cancer patients

Task 3 Development and optimization of antibodies for use on human tissue microarrays (TMAs)

Methods: anti-ER α , anti-Src IF: breast test array (antibody titer), ER α boutique array; Image capture with PM-2000 fluorescence microscope; AQUA; analysis of score frequency distribution & linear regression for reproducibility

Timeline: Months 17-22

Outcomes/Deliverables: ER α , Src, and “active” Src antibodies optimal for IF on full TMAs

Task 4 Assessment of the prognostic and predictive value of the non-genomic ER α pathway assay using a large patient cohort

Methods: IHC using anti-ER α , anti-Src, anti-“active Src” on full cohort TMA (majority with long-term follow up); Image capture with PM-200 fluorescence microscope; AQUA analysis; score frequency distribution, cut point analysis; Clinical data retrieval; Generation of Kaplan-Meier survival curves; Univariate and multivariate analyses; IF using anti-ER α , anti-Src, anti-“active Src” on special cohorts (300 patient Yale cohort and Swedish cohort, both with tamoxifen)

treatment); Image capture with PM-200 fluorescence microscope, AQUA analysis, Clinical data retrieval; Generation of Kaplan-Meier survival curves, Univariate and multivariate analyses

Timeline: Months 22-36

Outcomes/Deliverables: Determination of the prognostic and/or predictive value of non-genomic ER α pathway assay for breast cancer patients

The goal of Task 1 was to develop a model (cell lines grown in culture), which could be used (Task 2) to validate an assay that would test for genomic & non-genomic ER in actual patient samples (Task 3 & 4). The cell lines would therefore be developed & validated in culture, but with the goal of being spun into pellets and built onto a formalin-fixed, paraffin-embedded TMA. There, they would be stained alongside actual patient samples, in order to serve as the ideal control for a “non-genomic” tumor.

While beginning to culture cell lines and explore expression in patient tissue, I quickly realized that no single model would encompass all components of ER non-genomic signaling that may be of importance. The Her218 model was characterized by non-nuclear ER and high levels of HER2 (19), but this was only one mechanism of Tamoxifen resistance out of many that are possible in actual patients. I didn't want to bias my clinical assay to just one pattern I might find in my clinical samples. Furthermore, the validity of the Her218 model itself soon came into question as well. Once I actually began culturing the cell lines (Tamoxifen-resistant as well as long-term Estrogen-deprived), I was finding them extremely difficult to reproducibly maintain. I decided it would ultimately be more advantageous to examine the actual clinical samples first, to see if non-nuclear ER α was, in fact, detectable and measurable in human patients. If I could measure it, then I could go back to a preclinical model in order to validate the biology behind what I was seeing in patient tissue. Therefore, I began working towards the goals proposed in Tasks 2-4 of my proposal.

Validation of full-length ER antibodies (multiple epitopes) and development for use on TMAs (Tasks 2 & 3)

I began looking at full-length ER, both nuclear and non-nuclear, working with a panel of antibodies to different epitopes (N-terminal and C-terminal) of the receptor. I chose four monoclonal antibodies: 1D5 (mouse, N-terminal, the current clinical gold standard), F10 (mouse, C-terminal, shown to detect cytoplasmic ER in previous studies), SP1 (rabbit, C-terminal, shown to be equivalent or better than 1D5 for clinical analysis in recent years) (20), and 60c (rabbit, N-terminal, also shown to detect cytoplasmic ER in previous studies). In a panel of cell line controls, known to be positive or negative for ER, all antibodies showed specificity for full-length receptor (66kD), with equivalent sensitivity (**Figure1**).

I also used the above antibody panel to examine the localization of ER in cell lines, upon activation. This was done by growing cells on coverslips, stimulating +/- Estradiol (E2), performing immunofluorescent staining, and visualizing using the Delta Vision deconvolution microscope. This also served as further validation of *in situ* ER detection by the antibodies. I was able to confirm the specificity of staining and predominantly nuclear localization of ER α using all ER66 antibodies (SP1, F10, 60C, 1D5). Upon rapid stimulation with E2 (5 minutes), it appeared in some cases there was a shift in ER localization to the cytoplasm, however, the observed effects were time-sensitive and difficult to catch, as well as occasionally confounded by cell confluence (which also appeared to affect ER subcellular localization). Therefore, the ability of the antibodies to detect cytoplasmic ER was further confirmed by the use of MCF-7 cells that had been engineered to express a GFP-tagged “cytoplasmic-ER” which was lacking its Nuclear Localization Sequence (NLS). These cells were stained using the same protocol above, and compared to a wild-type ER control (GFP-nER) (**Figure 2**). Using F10, SP1 and 60c, I was able to visualize strong, specific cytoplasmic staining for ER (which co-localized

with GFP) in the GFP-cER cells, while seeing strong, specific nuclear staining (co-localized with GFP) in the GFP-nER cells.

The panel of antibodies was then developed for use on tissue microarrays, first by determining optimal titers for each using sequential cuts of breast test arrays, each containing 40 samples of both ER positive and negative patient tissue. Each antibody was then stained at its optimal titer on breast boutique arrays, containing 40 samples of patient tissue and about 15 cell line controls, each present in duplicate. Intensity of ER staining was quantified in the nuclear and non-nuclear/cytoplasmic compartment using AQUA technology. All antibodies to full-length ER showed similar distribution, both nuclear and non-nuclear, of AQUA scores, as well as good reproducibility between duplicate sets of scores. These frequency distributions and regressions can be seen for F10 and SP1 (two representative antibodies) in **Figure 3**. Reproducibility between second- and third-fold redundant stains was also very good for each antibody.

Lastly, the antibodies were all compared on a full cohort of breast cancer patients from our institution (YTMA 49, retrospective collection of ~690 patients). I found that all antibodies (whether against N- or C-terminal epitopes) were able to detect nuclear ER in patients (F10 being the most robust, and 60c being the least sensitive), and were all highly correlated (**Figure 4**). Non-nuclear expression of ER was assessed using an AQUA script I developed (which used DAPI and cytokeratin to refine a cytoplasmic compartment). Results showed that since there was often high nuclear expression of ER, it was difficult to precisely distinguish cytoplasmic from perinuclear staining (using a cytoplasmic compartment defined by DAPI). In a few cases (3 of 600), high cytoplasmic ER expression was detectable in patients, confirming the antibodies were able to detect non-nuclear receptor if it were present at substantial levels. Furthermore, because the detection of cytoplasmic receptor was common across multiple epitopes, it suggested it was at least in large part bona-fide, full-length ER, instead of shorter isoforms (6, 7) or other receptors for estrogen (21, 22) as others have argued. This has been a very important finding, and I have continued to see this (detection of cytoplasmic ER with both N- and C-terminal antibodies) in patient cohorts I have analyzed since (will be discussed in **Figure 8**).

However, in realizing we needed to determine a more precise and reproducible way to measure the presence of non-nuclear ER, we were first struck by the variability in quantification of nuclear ER itself.

Development of an assay to reproducibly quantify nuclear ER in patient tissue & assessment of current ER misclassification rate

We decided that with the technology utilized in our lab, we had the ability to develop a method to quantitatively and reproducibly determine a cutpoint for ER positivity. We did this by first converting an AQUA score to an actual concentration of ER (in cell line controls), and then using a set of case controls (analyzed by AQUA alongside the cell line controls) to reproducibly determine a cutpoint for ER positivity every time a cohort is analyzed.

I began this process by culturing cell lines with a range of ER expression: ER-negative, ER-moderate (such as BT474), ER-high (such as MCF-7), as well as ER-overexpressing (Puro9 cells, which were MCF7s with doxycycline-induced ER overexpression) (23). Cell pellets from each were spotted on two boutique arrays for measurement of ER by IF and AQUA, while in parallel, lysates were collected for measurement of ER by western blot. The same ER antibody (1D5, the clinical gold standard) was used for all assessments.

The lysates analyzed by western were run alongside a standard curve of pure recombinant ER protein (**Figure 5A**). ER expression in the western blots was quantified using Image J software. The standard

curve allowed me to determine a conversion between band intensity (using Image J) and pure pg of ER present in each sample. This allowed me to calculate the pg ER per μg total protein in each cell line. The same cell lines were spotted onto a boutique array (along with other cell line controls and 40 patient controls), stained using IF, and ER was quantified by AQUA. For each ER positive line, AQUA scores were plotted against the pg ER/ μg total protein, and a regression ($r^2 = 0.54$) was obtained (**Figure 5B**). This allowed an AQUA score to be converted to a concentration of ER for the set of 40 patient controls on the array. A cutpoint of positivity for ER in the Index of Control Cases was determined to be between 25-30 pg/ μg (**Figure 5C**), but using the known expression of ER in cell lines (western blot) further validated by visual analysis of images from IF staining (**Figure 5D**). These patient controls serve as the Index of Control Cases, and the slide containing them is stained alongside any patient cohort TMAs that are analyzed for ER in the future.

Currently, the clinical gold standard for measuring ER involves a brown stain and scoring by a pathologist (subjective). This method is not quantitative nor standardized, which has reached public awareness recently, due to an incident in Canada that revealed a high rate of false-negative classification there (24). There is much growing concern over this problem amongst clinicians and scientists (25, 26), and currently, there is very limited data on what the actual misclassification rate for current US practice may be. Since I have developed a quantitative assay for measuring ER as a continuous variable, I was then able to use this assay on our retrospective cohorts to expose the level of ER misclassification, as well as its significance.

I have performed the assay on a retrospective cohort from Yale with survival and recurrence information (YTMA 130), and compared ER classification by AQUA to pathologist performed conventional ER classification. We found a total misclassification rate of 20.1% (47 of 234 patients), with 19.7% of patients (46 of 234) misclassified as ER negative by Pathologist but ER positive by AQUA, and 0.4% of patients (1 of 234) misclassified as ER positive by a Pathologist but ER negative by AQUA. Representative images of ER immunofluorescent staining for each of these classifications can be seen in **Figure 6**, and a distribution of ER expression (in pg/ μg) by AQUA can be seen, stratified according to these classifications, in **Figure 7A**.

We were most interest in the patients misclassified as ER negative by a pathologist (presumably not given hormonal therapy), but classified as ER positive by AQUA, to see whether they showed survival behavior like true ER positives (would be potential candidates for therapy) or whether they behaved like true ER negatives (Pathologist classification was more accurate). We first showed that in this patient population, stratification by Pathologist classification alone (**Figure 7B**) or AQUA classification alone (**Figure 7C**) provided significant prediction of 10-year recurrence-free survival (RFS). When patients were stratified according to both classifications (**Figure 7D**), we saw that all 19.7% of AQUA-positive/Pathologist-negative patients (red and orange lines) showed 10-year RFS behavior more similar to true ER positives (pink line) than true ER negatives (blue line) on a Kaplan-Meier curve. This was a striking result, and **Figures 7E-G** show each of those curves on their own in contrast to the true ER negatives (significant or trending towards so, on account of small patient numbers).

This was a surprisingly high rate of disagreement between AQUA and a Pathologist, and the first concrete evidence anyone has been able to show thus far about current rates of misclassification in the US. Furthermore, our data suggests that patients who even have subtle levels of ER (detectable using our AQUA assay) do seem to behave like true ER positives, and should be treated clinically as such. This data (**Figures 5-7**) is currently being prepared for a paper we will try to submit to JNCI, given its clinical implications.

Development of an assay to quantify non-nuclear (cytoplasmic) ER in patient tissue & assessment of prognostic value (Task 3 & 4)

When analyzing ER expression on this retrospective cohort of 560 breast cancer patients (YTMA 130), I was able to detect a much higher rate of strong cytoplasmic staining for ER than on the first cohort (YTMA 49) I studied. Instead of analyzing a raw cytoplasmic score (which didn't account for high nuclear staining that often confounded results), we developed a "C-score" by subtracting the nuclear AQUA score (on a 100-pt scale) from the cytoplasmic score (on a 100-pt scale), and adding 100 (AQUAcyto - AQUAnuc + 100). A distribution of C-scores from this patient population is shown in **Figure 8A**, with representative images from various scores shown in **Figure 8B**. As mentioned earlier, this cytoplasmic staining was detected with both N- and C-terminal ER antibodies (**Figure 8C**), suggesting it was real, full-length receptor.

When using a cutpoint of 100 (**Figure 8A**) to isolate the patients with a higher amount of cytoplasmic ER than nuclear ER, we found a striking difference in 10-year RFS ($p = .0078$) on this patient cohort (**Figure 8D**). This is the first evidence to date (previously hypothesized in various preclinical models) of non-nuclear/cytoplasmic ER showing prognostic value (negative in this case) in actual clinical samples. We are very excited about this preliminary data and are in the process of confirming this on additional cohorts in order to further validate it.

Validation of antibodies to Src and pER and development for use on TMAs (Tasks 2 & 3)

Lastly, I have validated antibodies for Src and phosphorylated ER (pER), to potentially be used in future assays. There are multiple residues on ER whose phosphorylation has been suggested to play a role in non-genomic signaling. We chose to initially try the antibody used most frequently in publications, raised against the serine residue (Serine 118) on ER that is phosphorylated by MAPK and involved in non-genomic signaling (27). This antibody did, indeed, detect a specific band of expected size in MCF7 cells, which increased in response to rapid stimulation with E2 (**Figure 9A**), as expected during non-genomic signaling. The antibody was also stained on cells grown on coverslips and stimulated under the same conditions, and the expected punctuate staining (both nuclear and some cytoplasmic) was seen with high specificity (**Figure 9B**). An antibody to total Src was concurrently stained on the stimulated cells, and also showed beautiful and specific staining by IF, in an expected membranous/cytoplasmic pattern (**Figure 9B**).

The Src antibody was then optimized for staining on TMAs by titrating on breast test arrays and staining on a breast boutique array. Frequency distributions as well as regressions showing reproducibility can be seen in **Figure 10A**, along with representative IF images of high and low levels of Src (**Figure 10B**), showing specific staining on patient tissue. This antibody is currently ready for further development into a multiplexing assay with full-length ER. The same distribution and regression analysis was done for the pER antibody (**Figure 11A and B**), looking at both nuclear and cytoplasmic localizations of the receptor. Expression of pER was compared to total ER on the same boutique array (**Figure 11C**), and we were slightly concerned by the finding that some patients appeared to have moderate or high levels of pER, but low to negative levels of total ER (example in bottom panel, **Figure 11D**). Current evidence in our lab also suggests that phospho-antibodies are subject to variation due to differences in tissue time-to-fixation (antigenicity deteriorates on tissue resections, which are not immediately fixed, in comparison to core biopsy specimens). Indeed, in one set of patient tissue I received (resection paired with a core biopsy), I saw decreased pER expression in the resection. Given that most of our cohorts (and cohorts we obtain) contain both resections and biopsy specimens, we are weary of continuing development of this pER assay at the current time.

KEY RESEARCH ACCOMPLISHMENTS:

- Validated that four different monoclonal antibodies against multiple epitopes of full-length ER (1D5 the clinical gold-standard, as well as F10, SP1, and 60c) are specific and generally equivalent in their detection of nuclear ER by western blot, by IF on cell lines, and by IF on tissue microarrays.
- Validated that all four monoclonal antibodies above are able to detect non-nuclear/cytoplasmic ER, and with similar sensitivity.
- Developed an assay to reproducibly quantify nuclear ER in patient tissue (in pg/μg total protein) using an Index of Control Cases.
- Used the assay above to assess the ER misclassification rate in clinical samples, revealing a population of patients currently classified as ER-negative, who actually have low levels of ER and show survival behavior like true ER-positive patients.
- Confirmed that the majority of non-nuclear ER found in patient tissue thus far appears to be full-length receptor (not shorter isoforms or cross-reactive epitopes)
- Developed an assay and scoring method to measure non-nuclear/cytoplasmic ER, which appears to have negative prognostic value.
- Validated an antibody against phosphorylated ER (Serine118) for specificity by western and by IF in cell lines, but confirmed it is not yet valid for IF use on TMAs (evidence to suspect expression is subject to variation based on tissue time-to-fixation).

REPORTABLE OUTCOMES:

Abstract & Poster Presentation: 2009 American Society of Clinical Oncology (ASCO) Annual Meeting

Evaluation of the false-negative rate of standardized and quantitative measurement of estrogen receptor (ER) in tissue using AQUA technology (#567 Booth #2679)

Abstract & Poster Presentation: 32nd Annual San Antonio Breast Cancer Symposium, December 9-13, 2009

Development of a quantitative and standardized assay to measure ER protein concentration in breast cancer tissue & improve current patient misclassification (#4068)

Presentation: Yale University Department of Pathology Research in Progress.

CONCLUSION:

In conclusion, my results to date have allowed me to develop a quantified and reproducible assay to measure nuclear ER, and use this assay to assess the rate and significance of misclassification in breast cancer patients today. This has enormous implications for the way we classify patients in the future (in fact, ASCO-CAP is very likely to re-define ER testing guidelines to include *any* ER staining as positivity). Furthermore, the board at ASCO-CAP was interested in our method for quantifying and standardizing ER measurement (requested us to send a detailed protocol), which even if not used in any future guidelines, has provided important conceptual ideas for ideal ways to standardize in the future. The second major result we have found is the detection of cytoplasmic ER in actual patients, and the confirmation that it appears to be full-length ER (as opposed to shorter isoforms or an artifact of epitope cross-reactivity). Patients with higher levels of cytoplasmic ER in relation to nuclear ER appear to have worse prognosis, as our hypothesis would predict, suggesting that the biology behind non-genomic signaling may in fact play a role in actual patients. This is the first clinical evidence to date on the presence and prognostic value of cytoplasmic ER, and thus we are very eager to reproduce this data and hopefully put together a publication in the near future. I am also going to continue developing the assay to multiplex Src with full-length ER and determine whether this can provide additional prognostic or predictive value over ER alone.

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APPENDICES & SUPPORTING DATA:

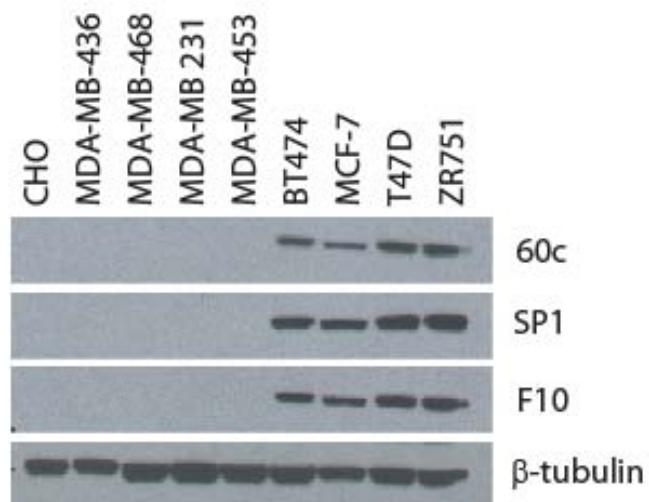


Fig 1. N- and C-terminal ER antibodies are specific and equivalent by western blot. Full-length ER (66kD) was detected in whole cell lysates from a panel of cell line controls (BT474, MCF-7, T47D and ZR751 known to be positive, the rest negative) using three different monoclonal antibodies directed against the receptor: 60c (rabbit, N-terminal epitope), SP1 (rabbit, C-terminal epitope), F10 (mouse, C-terminal epitope). All antibodies appear equivalent in their specificity. Beta-tubulin is shown as a loading control.

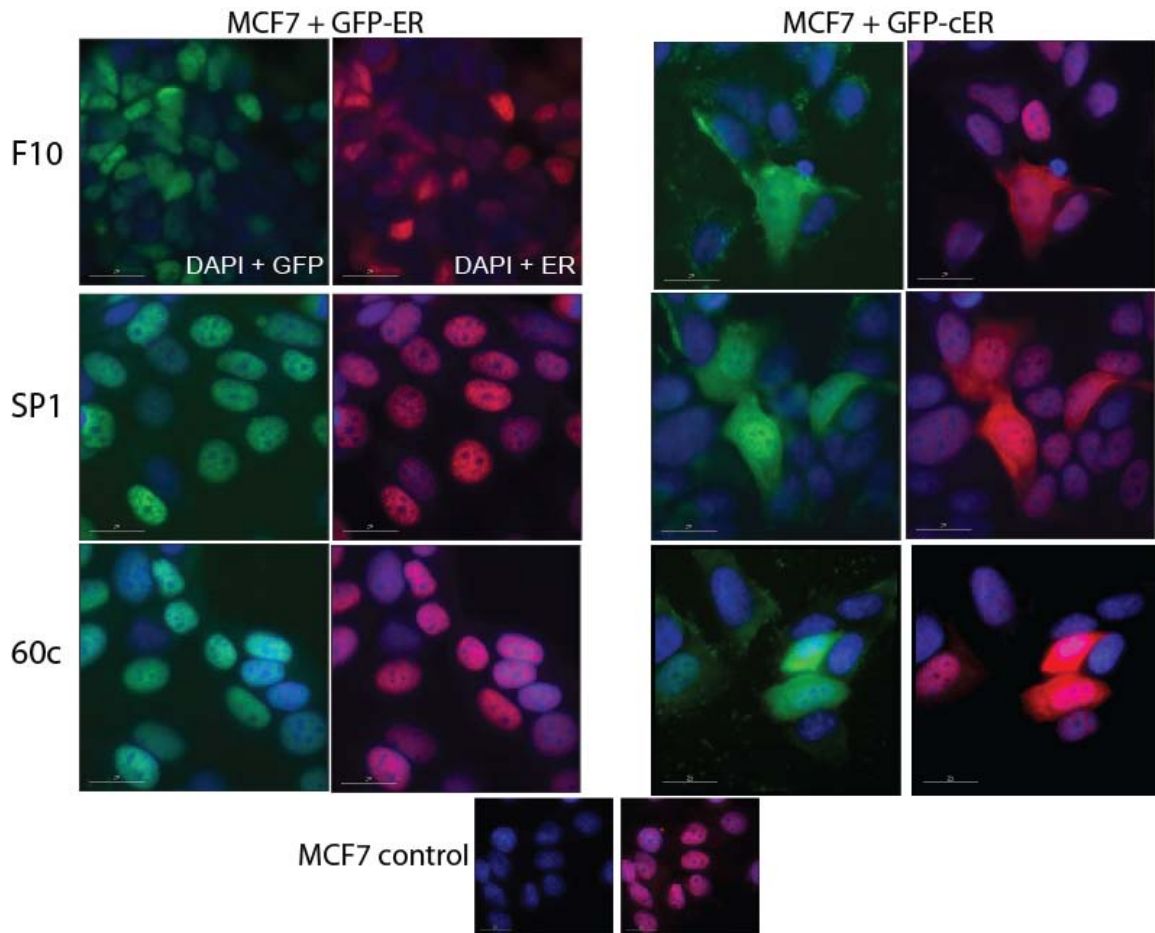


Fig 2. N- and C-terminal ER antibodies are specific and equivalent by IF on cultured cell lines, and can recognize both nuclear & cytoplasmic localizations of ER. MCF7 cells were grown and stably transfected with a GFP-tagged wild-type ER (MCF7 + GFP-ER) or a GFP-tagged cytoplasmic ER (cER), which was a deletion mutant lacking its nuclear localization sequence (MCF7 + GFP-cER). These cells were cultured, grown on coverslips, fixed, and stained using immunofluorescence with three antibodies to the N- and C-terminus of ER (shown in red), along with DAPI and GFP. All three antibodies (F10 and SP1 C-terminal, 60c N-terminal) were able to recognize strong, specific nuclear staining for ER (co-localized with GFP) in the GFP-ER cells (red, left panels). All three were also able to recognize strong, specific cytoplasmic staining for ER (also co-localized with GFP) in the GFP-cER cells (red, right column of panels). Endogenous ER (red) can be seen in MCF7 controls (bottom) untransfected with a GFP-tagged construct.

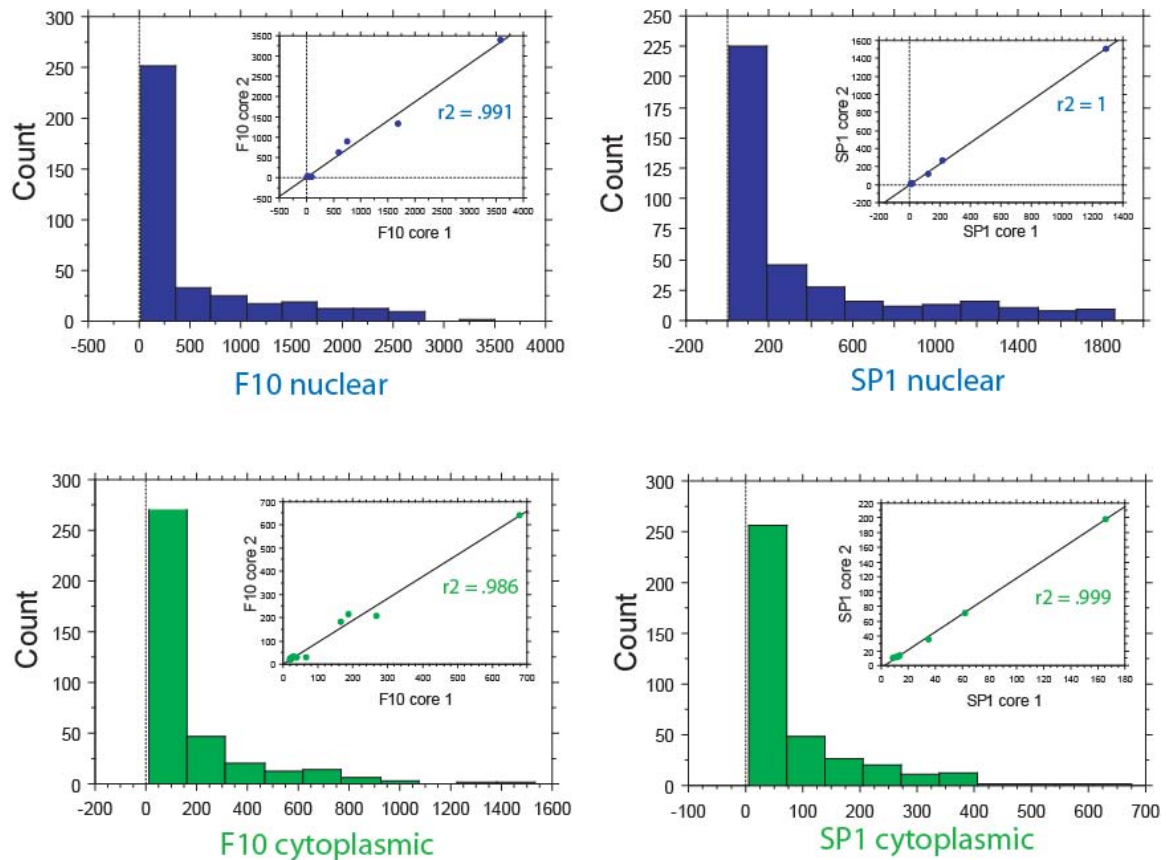


Fig 3. Score frequency distribution and linear regression for antibodies to full-length ER. All monoclonal antibodies against different epitopes of full-length ER were titrated on breast test arrays and stained at their optimal titer on boutique arrays to assess reproducibility. They were then stained on a full cohort of breast cancer patients from Yale (retrospective collection). Distribution of nuclear scores (top panels) and non-nuclear/cytoplasmic scores (bottom panels) are shown for two of the antibodies, F10 (mouse, $n = 379$, left panels) and SP1 (rabbit, $n = 381$, right panels). Linear regression between duplicate cores from 8 pairs of cell line controls are shown in the insets for each.

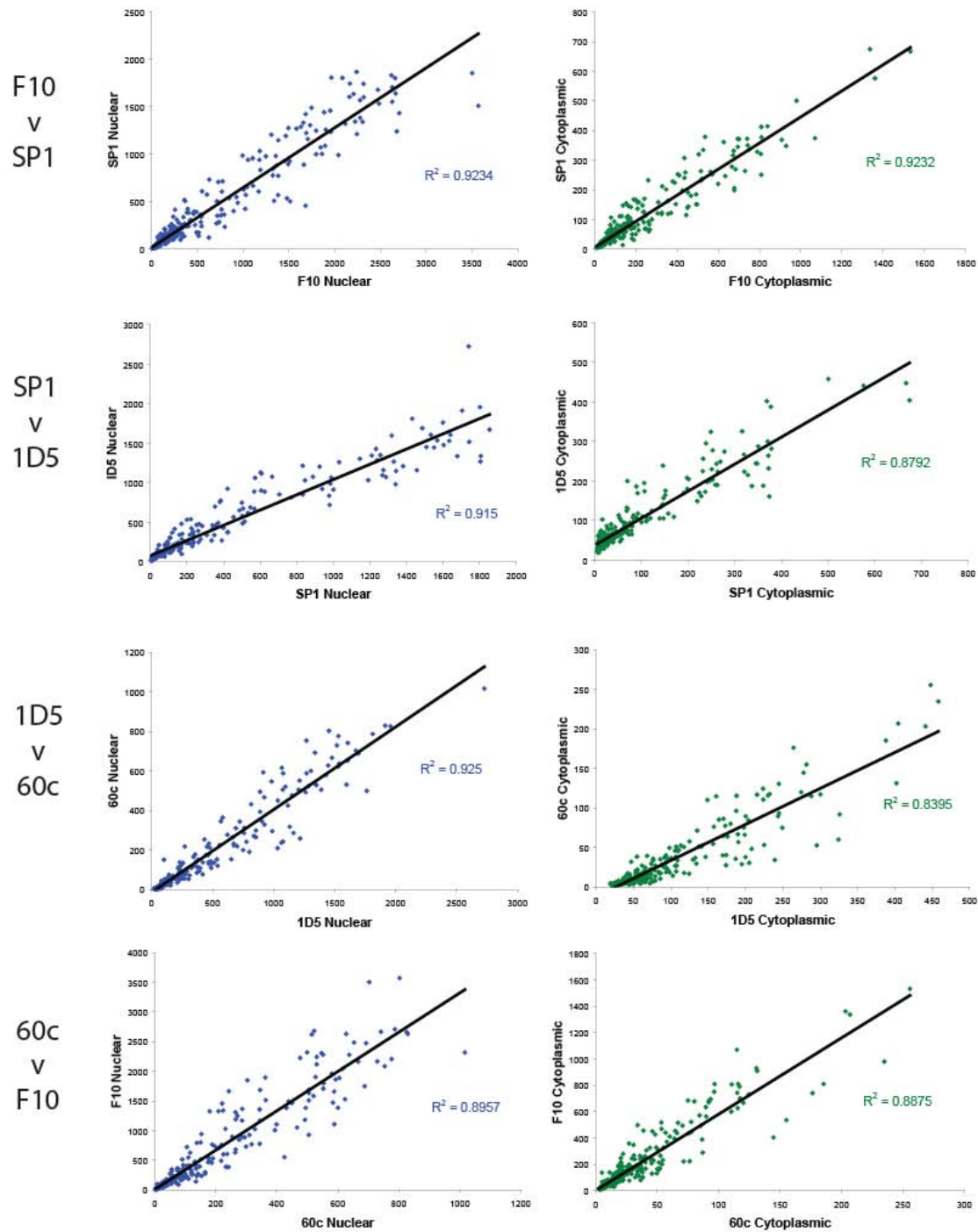


Fig 4. N- and C-terminal antibodies are equivalent in their recognition of both nuclear and cytoplasmic ER, when using immunofluorescence on formalin-fixed paraffin-embedded TMAs. Sequential cuts of a tissue microarray containing 375 breast cancer patients were stained using immunofluorescence using four monoclonal antibodies to full-length ER: F10 (C-terminal, mouse), SP1 (C-terminal rabbit), 1D5 (N-terminal mouse), 60c (N-terminal rabbit). Expression of ER in the Nuclear (blue) and Cytoplasmic (green) Compartments was quantified using AQUA and regressions are shown between the distribution of AQUA scores for each antibody. The r-squared value averaged 0.915 for regression between nuclear ER staining with different antibodies, and 0.882 for regression between cytoplasmic ER staining.

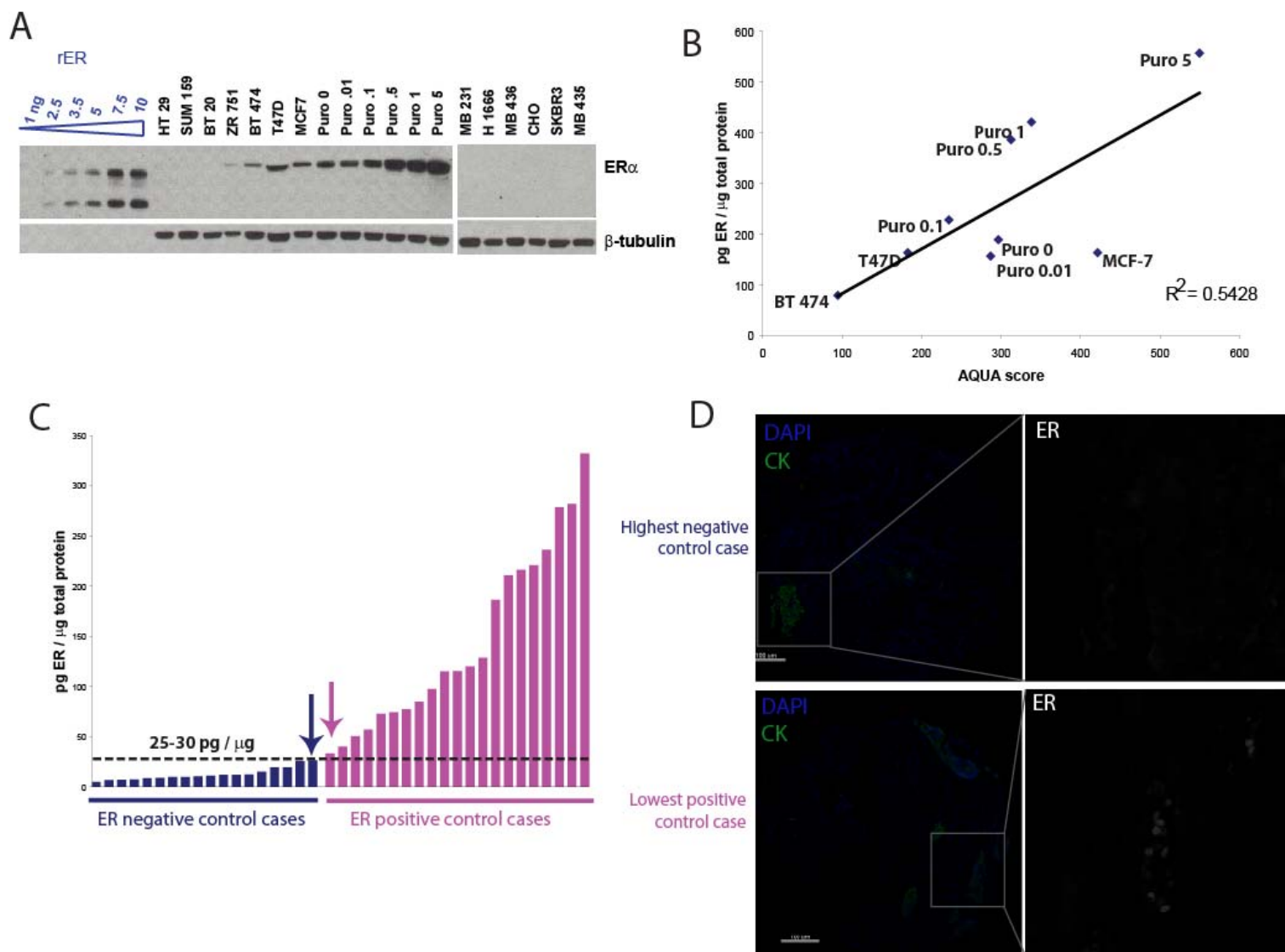


Fig 5. Quantification of ER (in pg/μg total protein) in an Index of Control Cases, which are used to determine the cutpoint for Nuclear ER positivity. A) ER was measured in cell line controls by western blot alongside recombinant ER (rER) to determine absolute concentration in pg/μg total protein. B) The absolute concentration of ER (pg/μg) was then correlated to the amount of ER quantified using AQUA (and IF staining) in the same cell line controls. C) The regression in B was used to determine a concentration of ER from the AQUA scores of a set of case controls present on the same TMA as the cell line controls. The distribution of ER in this Index of Control Cases is shown, with a cutpoint of positivity at 25-30 pg/μg. D) Immunofluorescence AQUA images of ER in the highest negative control case (blue arrow in C) and the lowest positive control case (pink arrow in C) are shown to validate the cutpoint.

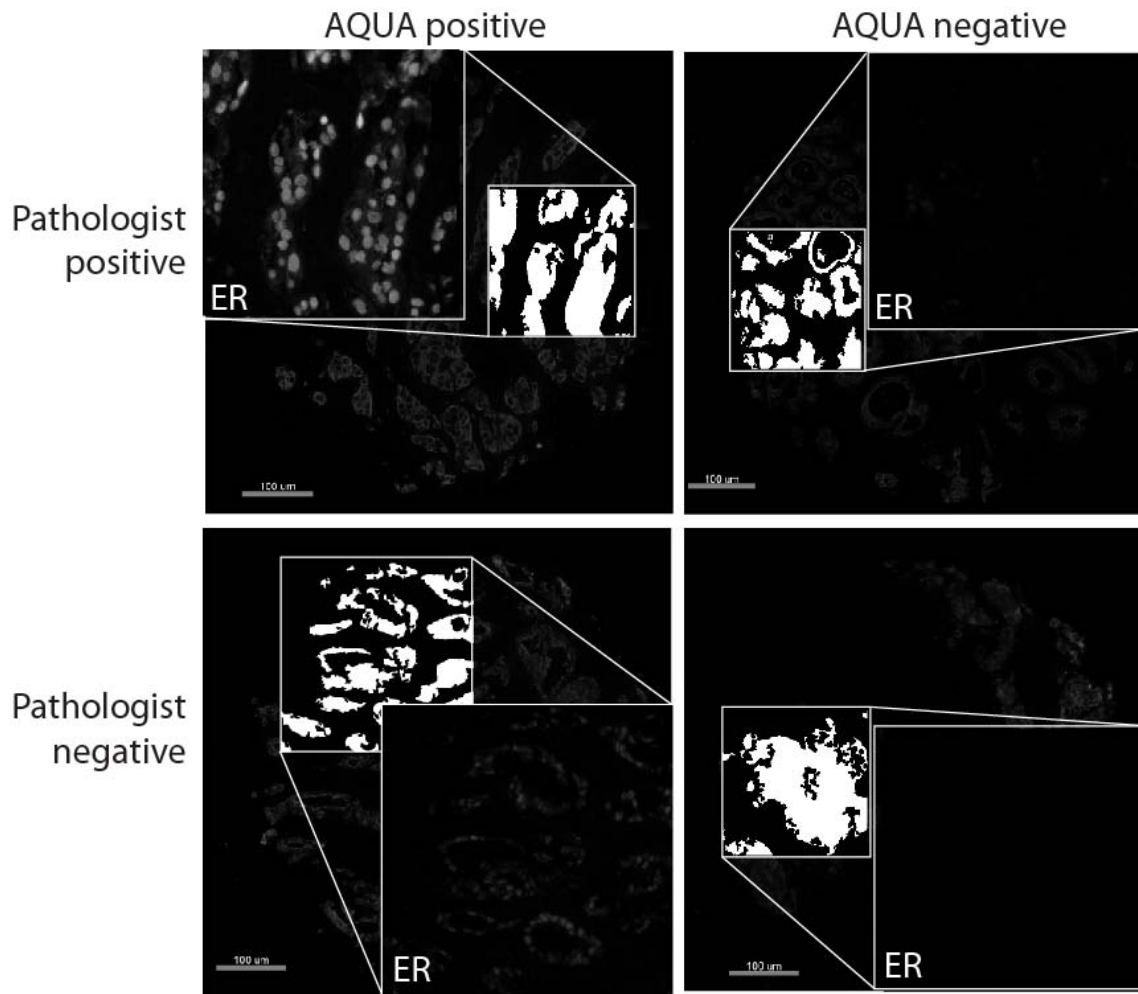
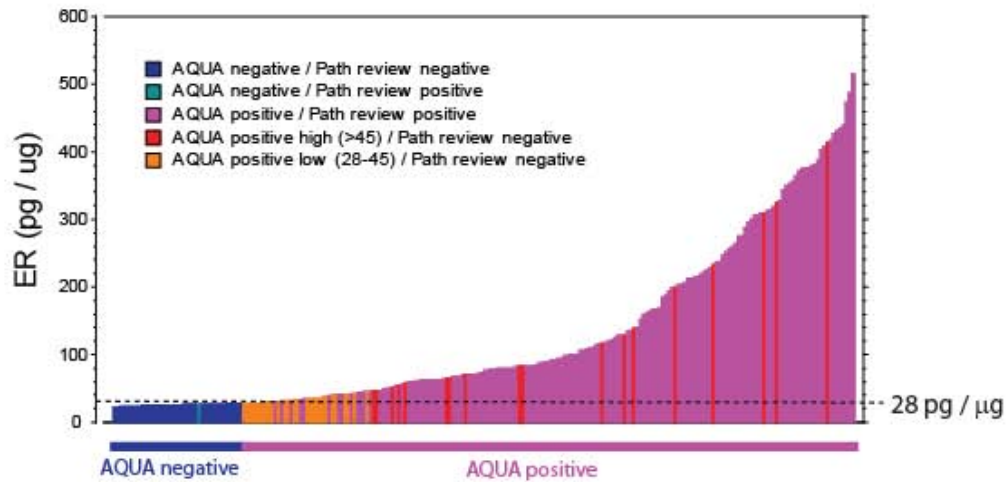
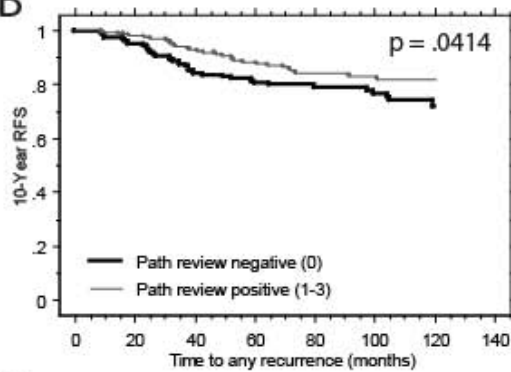


Fig 6. Representative images of ER immunofluorescent staining in 4 patients classified as ER-positive or –negative by a Pathologist or by AQUA. In a tissue microarray containing 560 breast cancer patients (retrospective cohort), ER status (positive or negative) was determined by both a Pathologist reviewer (using IHC, where 1-3 is positive and 0 is negative) and by AQUA (in pg/μg, using IF and a cutpoint of 28 pg/μg determined as shown in Fig 4). Immunofluorescent images of ER staining for patients where AQUA status agreed with Pathologist status are shown (top L, bottom R), as well as images of an AQUA positive/Pathologist negative patient (bottom L), and an AQUA negative/Pathologist positive (top R).

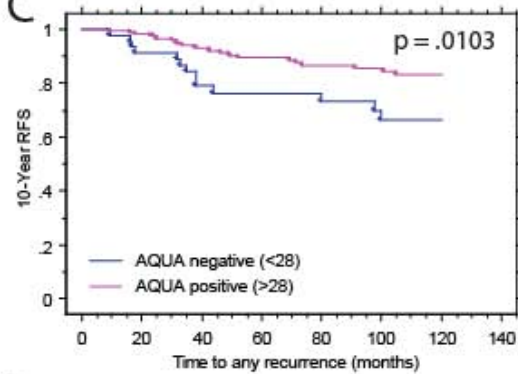
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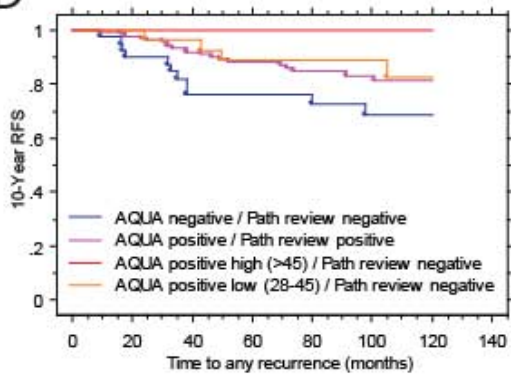
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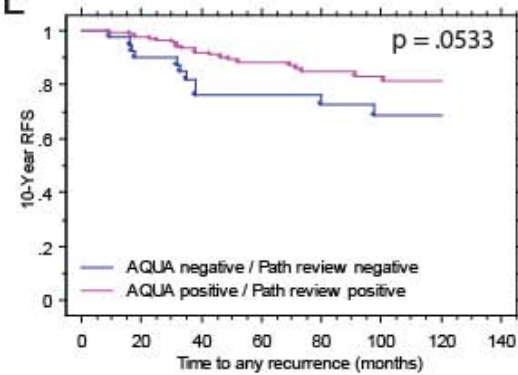
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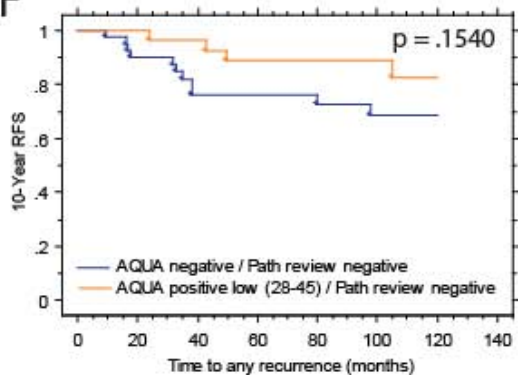
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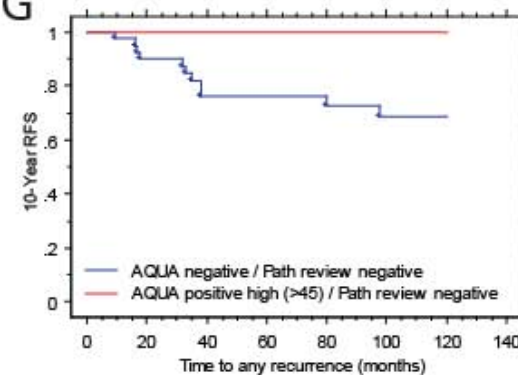


Fig 7. Misclassification of ER status in YTMA 130 Cohort. In a tissue microarray containing 560 breast cancer patients (retrospective cohort), ER status (positive or negative) was determined by both a Pathologist reviewer (using IHC, where 1-3 is positive and 0 is negative) and by AQUA (in pg/ μ g, using IF and a cutpoint of 28 pg/ μ g, determined as shown in *Fig 4*). A distribution of ER by AQUA (pg/ μ g) is shown in *A*, where each case is color-coded according to its ER status by both AQUA and a Pathologist. The misclassified cases were of greatest interest, with one patient considered negative by AQUA but positive by the Pathologist. The cases classified as ER positive by AQUA but negative by the Pathologist were further subdivided into low AQUA positive (28-45 pg/ μ g, orange) and highly AQUA positive (>45 pg/ μ g, red). *B-G* show representative Kaplan-Meier curves for analysis of ten-year recurrence-free survival (RFS). In *B*, patients are grouped according to Pathologist status alone ($p = .0414$), while in *C*, by AQUA status alone ($p = .0103$). In *D*, patients are grouped according to the classification shown in *A*, with the single AQUA-negative/Pathologist-positive case being discarded. It is clear that the cases considered positive by AQUA but negative by the Pathologist (orange and red) do indeed behave like the true ER positives (pink), rather than the true ER negatives (blue). *E* shows the marginal significance in RFS when comparing the true positives to the true negatives alone. *F* and *G* show the survival pattern of the AQUA positive/Pathologist negative patients to be different than that of true negatives, whether low ER by AQUA (*F*, trending towards significance) or high ER by AQUA (*G*, no p -value due to zero events).

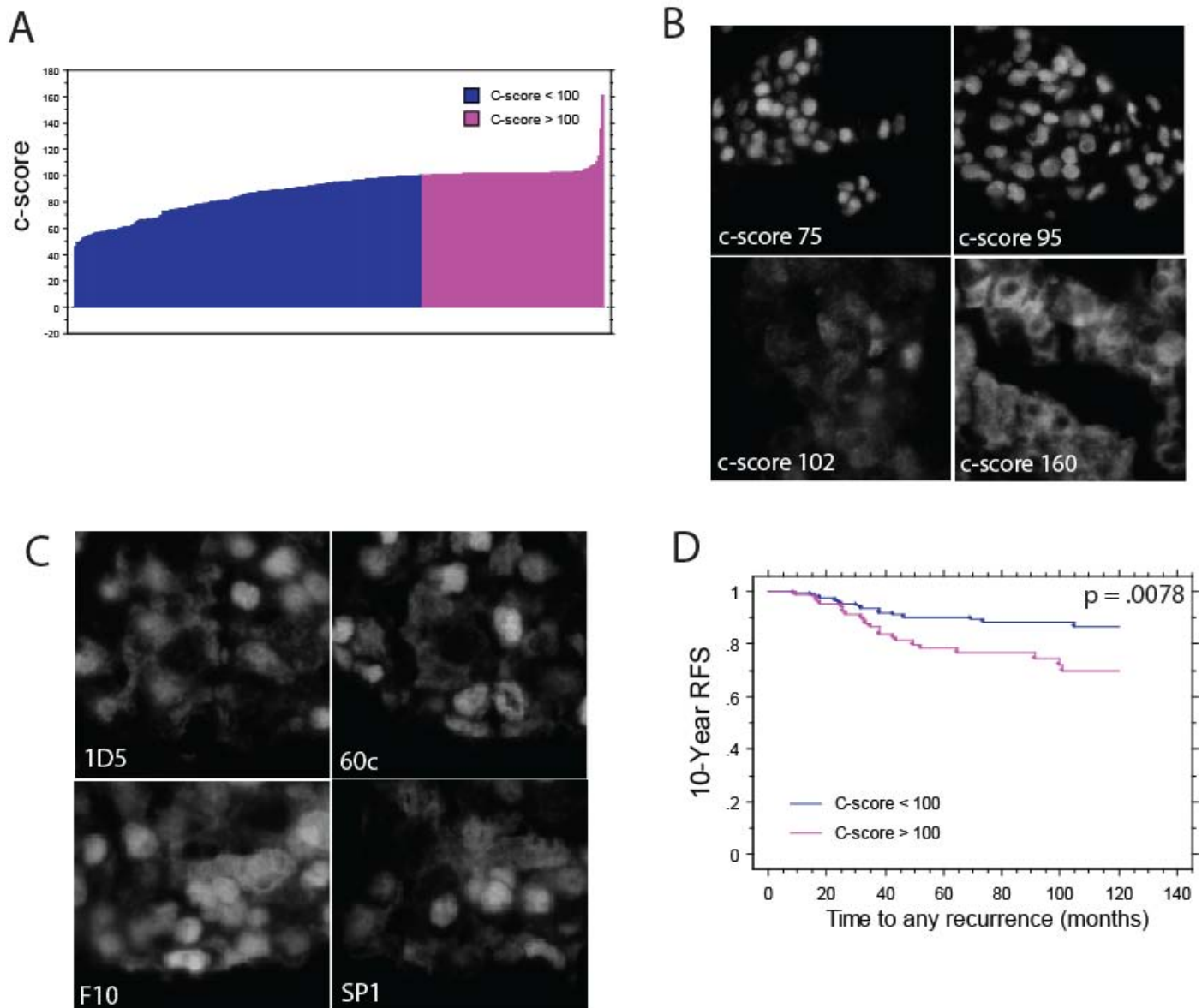


Fig 8. Significance of non-nuclear (cytoplasmic) ER localization in breast cancer patients, using antibodies to multiple epitopes (N- and C-terminal). Non-nuclear ER was quantified using IF and AQUA in a retrospective cohort of 560 breast cancer patients (same as in Fig 5 and 6), by using a C-score (AQUAcyto-AQUAnuc +100), where AQUAcyto is the AQUA score of ER in the cytoplasmic compartment on a 100-pt scale, and AQUAnuc is the AQUA score of ER in the nuclear compartment on a 100-pt scale. **A**, Distribution of C-scores for patients in this cohort (using SP1 antibody), showing a cutpoint of 100. **B**, Four C-score examples with the ER antibody (SP1) used in this analysis. **C**, Four representative images of a patient with non-nuclear ER, showing detection with both N-terminal and C-terminal antibodies. **D**, Kaplan-Meier survival analysis showing ten-year RFS for patients grouped according to ER C-score ($p = .0078$) cutpoint shown in **A**.

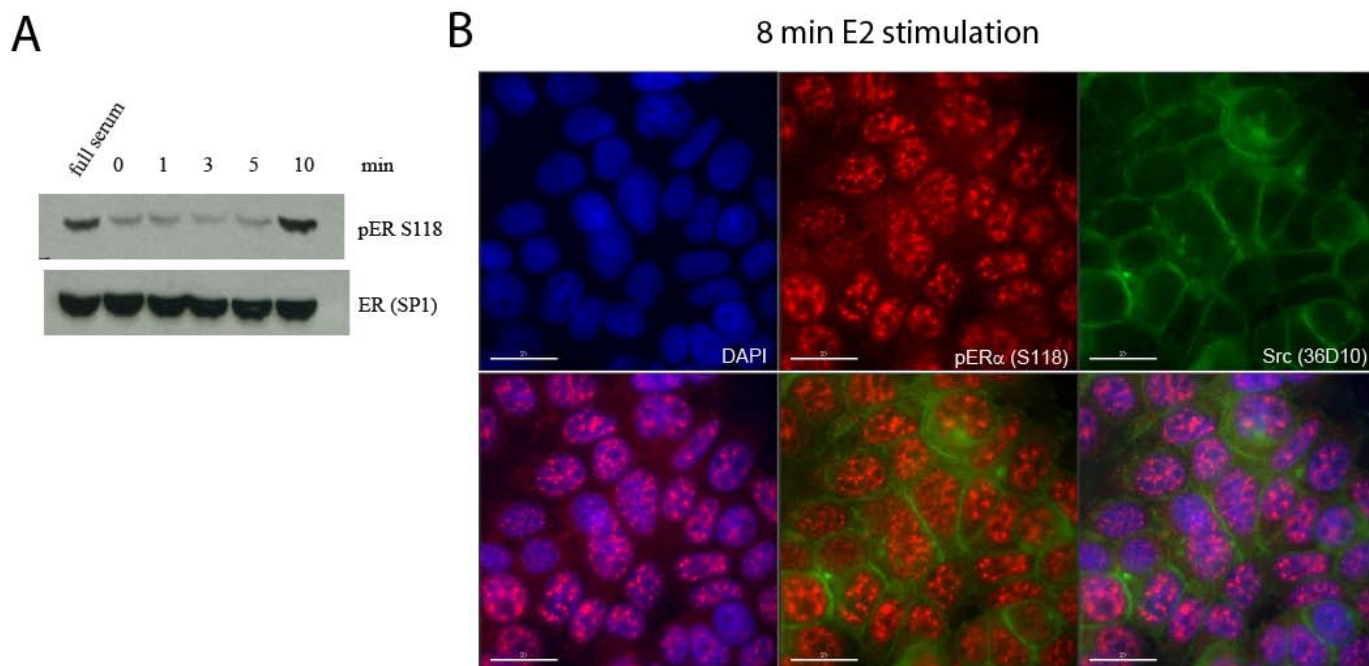


Fig 9. Validation of Src and pER antibodies by western blot and immunofluorescence (IF) in cell lines. *A*, Whole cell lysates from MCF-7 cells that were serum-starved for 24 hours before stimulation with 10nM E2 for 0, 1, 3, 5 or 10 minutes was resolved on SDS-PAGE gel and blotted using the pER antibody (S118) compared to total ER (SP1). Rapid increase in phosphorylation was seen upon E2 stimulation, as expected. *B*, MCF7 cells were cultured on coverslips, serum-starved for 24 hours, then stimulated with 10nM E2 for 8min before being fixed and stained using IF with antibodies to pER (red, serine 118), and Src (green, clone 36D10). Merged images show specific staining as expected for both (punctate nuclear and cytoplasmic for pER, and membranous/cytoplasmic for Src).

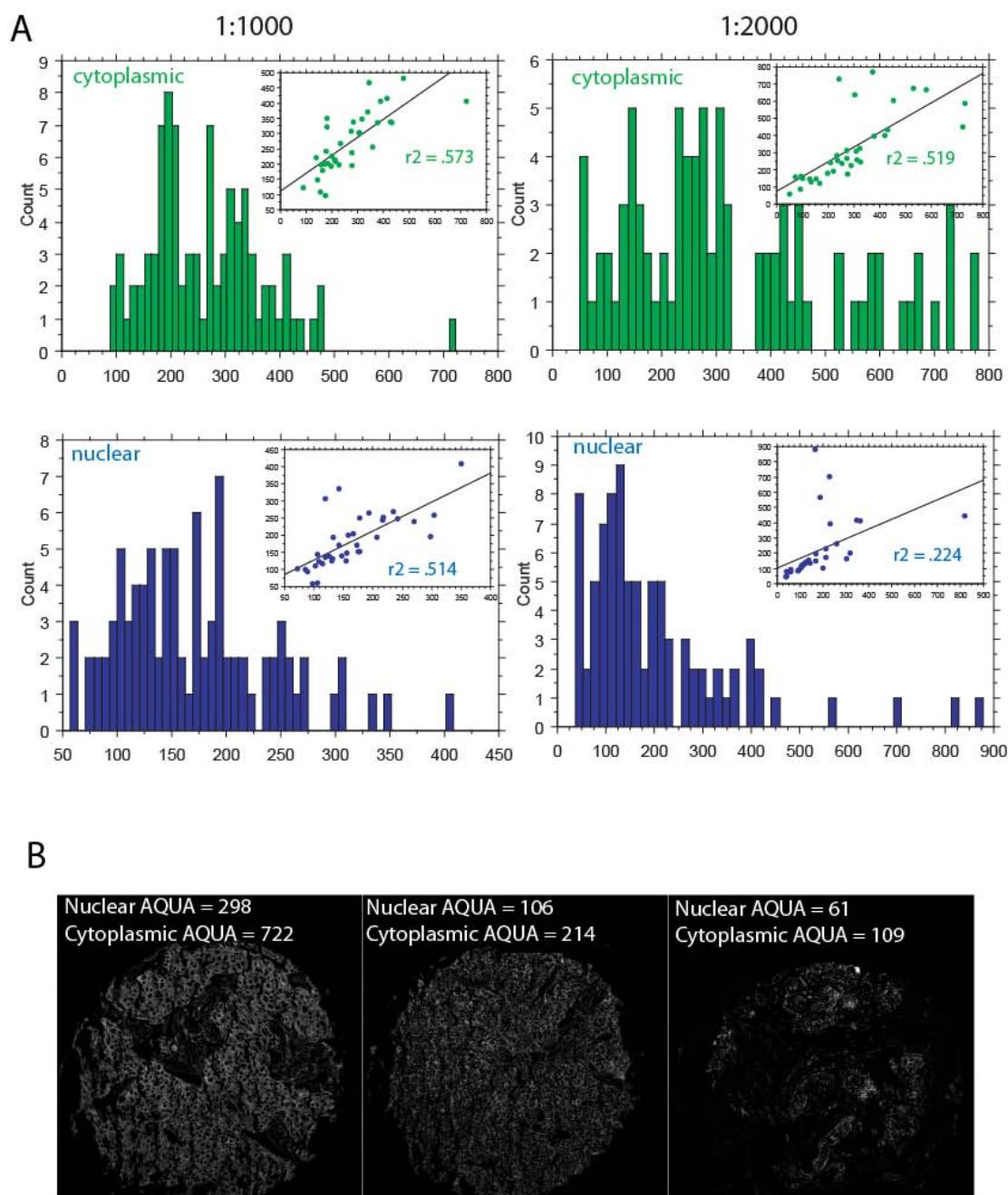


Fig 10. Optimization of Src antibody (36D10) for use on tissue microarrays. To optimize the Src (36D10) antibody for IF staining on tissue microarrays and quantification by AQUA, it was titrated on a breast test array and then stained at the two potential optimal titers on a breast boutique array containing 40 patients and 15 cell line controls. **A**, Frequency distributions of both cytoplasmic (green, top panels) and nuclear (blue, bottom panels) AQUA scores for Src expression are shown at both titers (1:1000 left panels, 1:2000 right panels), with regression between two duplicate cores shown in the insets. **B**, Representative images of a high expressor (left panel), medium expressor (middle panel), and low expressor (right panel) are shown at the optimal titer of 1:1000, with AQUA scores inset.

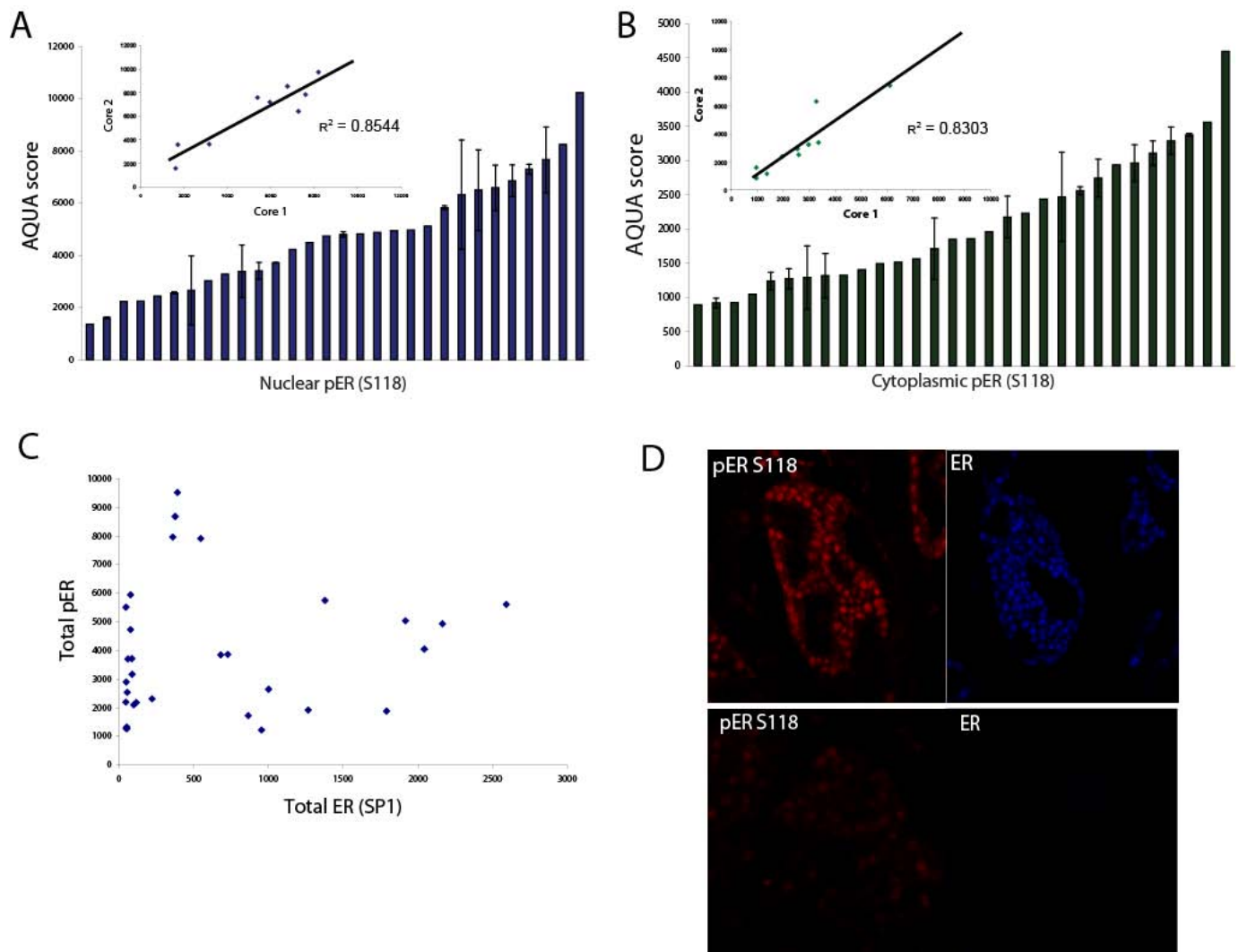


Fig 11. Optimization of pER antibody (S118) for use on tissue microarrays. To optimize the pER (Serine 118) antibodies for IF staining on tissue microarrays and quantification by AQUA, it was titrated on a breast test array and then stained at the two potential optimal titers on a breast boutique array containing 40 patients and 15 cell line controls. *A*, Frequency distribution of nuclear pER AQUA scores in the patients with adequate tissue, with regression between two duplicate cores shown in the inset. *B*, Frequency distribution of non-nuclear/cytoplasmic pER in the patients with adequate tissue, with regression between two duplicate cores shown in the inset. *C*, Representative images of a high expressor (top panel) and mid-to-low expressor (bottom panel), with pER (S118) shown in red and total ER (SP1 antibody) shown in blue. *D*, Correlation between pER and total ER (SP1) amongst the patient and cell line controls on the boutique array, showing suspicious levels of pER in spots with very little total ER.